IN THE U.S. PATENT AND TRADEMARK OFFICE

APPLICANT : Chennur Radhakrishna Reddy *et al*

SERIAL NO.: 09/656,561 GROUP : 1661

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FOR : AN IMPROVED PROCESS FOR CULTIVATION OF ALGAE

DECLARATION REGARDING NON-OBVIOUS NATURE OF THE PROCESS FOR CULTIVATION OF ALGAE OF THE INSTANT APPLICATION

Honorable Commissioner of Patents
Washington, D.C. 20231

Sir,

I Kalpana Harish Mody, am the main inventor of the above-stated patent application. I hereby declare that

I am a doctorate in the field of Bioscience. I completed my doctorate from Sauraushtra University, India in the year 1979. Right now, I am pursuing my research work in the field of Marine algal at Central Salt and Marine Chemicals Research Institute (CSMCRI), Bhavnagar, Gujarat INDIA, since the year 1978.

I have worked with aforementioned institute from year 1978 to till date.

My area of expertise is Marine algal and Marine Microbiology. I have more than 35 publication in this field. Also, I have won several awards like C.S.I.R Biotechnology Award in year 2001.

The institute, CSMCRI is an extremely reputed institute in the country/world and specializes in the field of Marine algal. The institute was established in the year 1954. The institute has infrastructure to conduct several complex and high technological experiments.

The Examiner has rejected claims 1 -18 under 35 U.S.C. 103(a) as being unpatentable over Dawes et al. in view of Mairh et al, which is my own publication.

I strongly feel that the instant Application is totally non-obvious against the cited arts. There is no clue to combine these two inventions and reach the invention of the instant Application.

I state that the prior art drawback resulted in the decrease of the daily growth rate of the alga grown in open waters compared to those grown in open waters without polythene bags. So, how can it motivate us to take reference of the same and reach the invention of this Application.

The bag method of cultivation has distinct advantages but the grown rate is definitely much lower than for open cultivation. This assertion is based on my comparative studies carried out in this location using both the methods and under otherwise identical conditions. Moreover the studies carried out for all seasons and the differences persist. Since farmers would be reluctant to undertake cultivation of the seaweed in the absence of a minimum acceptable growth rate such as that found for open cultivation with the conventional seaweed. My aim was to achieve the observed growth rate of the conventional plants in open cultivation while combining advantages of bag cultivation. The tissue culture plants enable us to achieve this minimum acceptable growth rate in bag cultivation and this is what I wish to claim. The prior art of Mairh et al. Does not bring out clearly the limitation of the bag process with regard to growth rate.

I state that the main drawback of micro-propagation is that progeny will possess only parental features and does not have a pronounced advantage over the parental populations in expression of desired traits (paper number 8, p.6).

Although the claimed method is a kind of asexual reproduction, it resulted in selection of a soma clone expressing consistently improved trait for growth from tissue cultured progeny in field cultivation in the sea. In the present invention, several clones were generated from single callus source. Of the several clones, 22 tissue culture clones were transferred to the field and selected one with two-fold increase in biomass over control plant in 60 days period in polythene bas method. The improved growth in this clone has been found to be stable and consistent even after two years of monitoring in the field cultivation in the sea. Further, the tissue cultured plants obtained in the present invention were indeed regenerated from "single cells" through micropropagules from pigmented uniseriate branched filamentous callus without the use of any plant growth regulator supplements to culture medium.

In the report by Dawes et al, the tissue culturing is done through propagation of micro cutting of 1-5 mm size (p.250) explants selected from main axis and thus the resulting plants will necessarily be the same as the parent. Further, the plant regeneration from callus was practically obtained from mass tissues by culturing 2 mm callus pieces in sterile liquid cultures with plant growth regulator supplements. Hormone induced traits are transit and they are normally expressed for limited period.

Mairh et al. reference does not tissue culture plantlets and did not used them in open sea conditions. I strongly feel that the present invention essentially describes an improved process for cultivation of algae wherein a high yielding tissue culture clone was grown in a bag and produced a biomass as much as that obtained for conventional variety in open waters without a bag (see example 7). Further, I additional cultivation experiments carried out without bag in the field in open waters revealed a two-fold increase in biomass of tissue cultured plant over conventional variety under identical culture conditions, substantiating the inherent superiority of the tissue-culture plant.

I respectfully submits that the Dawes did report filamentous callus for *Eucheuma denticulatum* and mainly bubbly type of callus for *Kappaphycus alvarezu*. Their studies invariably used culture medium with plant growth regulator supplements for growing either micro-propagules, callus or for regeneration of plantlets from callus.

However, the present invention demonstrates callus induction and plant regeneration via micropropagules, which Ire produced from somatic embryo-like structures from filamentous calli in a normal agar culture medium without use of any plant growth regulators (see example 2). In order to increase the production of micropropagules (Fig. 4), I have practiced a new culture method namely "embedded culture" wherein thin slices of callus pieces were implanted inside the agar (0.4%) and grown further (example 3). This method facilitated rapid growth of filamentous callus and led to the finding of development of somatic embryo-like structure in filamentous callus (Fig. 6). Supplementation NAA and BAP to embedded cultures enhanced the growth of callus as Ill as the process of formation of somatic embryo-like structure substantially in filamentous callus (example 4). The embedded culture method described in the present invention is novel and inventive in propagation of algal callus.

Dawes et al method suggests the possibilities of selection of new strains, it is clear from their subsequent work (Table -2 p.23, Journal of Applied Phycology 6:21-24, 1994) that the growth of such new strains in field trials did not slow significant difference in biomass as Ill dally growth rate between callus regenerated plants and normal plants. However, I observed a significant enhancement in the biomass between tissue-cultured plants compared to normal plants grown under similar conditions. I attribute this observation to somaclonal variation, as this consistently should improved growth rate even after cultivating for extended period in the sea.

In the present invention, first, I have demonstrated development of micropropagules (Fig. 3 and 4) directly from excised callus subcultured on agar plates.

For obtaining plant regeneration from callus, Dawes cultured cut calli pieces (2mm) in sterile liquid cultures with plant growth regulation supplements as described for agar substrate (p.250). In the present invention, I have never cultured callus in liquid cultures and only at micro-colony level, they were transferred to enriched seawater medium (without any plant growth regulators) to achieve rapid morphogenesis and plant regeneration.

Since, the propagules developed from subcultured callus could regenerate and develop into full plants morphologically similar to farm plants water transplantation in the sea, the subsequent work was undertaken to increase the production of microphogenesis in callus. While screening the subcultured calli under microscope, I could observe some parts of callus found growing penetrated inside the agar and having darkly pigmented micro colonies of cells (4-10cells) similar to initial stages of somatic embryos of higher plants. This observation led us to develop a new method of growing callus pieces inside the agar (0.4%) plants. This has facilitated luxuriant growth of filamentous callus and thereafter helped to produce innumerable micro-colonies from micropropagules have come into

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existence after transferring into liquid cultures. Further, addition of plant growth regulators (NAA and BAP) to culture medium enhanced the growth as well as micro colony formation in filamentous callus that is inside the agar plants. To the best of my knowledge this is inventive and thus, patentable.

Other differences approaches between cited arts and present invention are as given below to show inventive nature of the invention

1. Morphogenesis of plants from calli Ire reported in liquid culnres enriched with different plant growth regulators similar to those used for callus induction.

However, in the instant Application morphogenesis of plants in the present invention was achieved from tiny micropropagules that grew independently from callus that was grows on agar plates Further the culture medium as never supplemented with plant growth regulator for callus induction purpose.

2. The term "micropropagules" was used to refer to micro-cuttings of plant pails

However, in the instant Application the term "micropropagules" was used to refer to the tiny micropropagules developed on about 60-70 days old subcultured callus.

3. There is no mention about development of somatic embryo-like structures from callus.

However, in the instant Application the micropropagules described in the present invention Ire found to develop from somatic embryo-like strucures from pigmented callus.

4. There is no information on enhancement of micropropagules production

However, in the instant Application, the micropropagules production was enhanced by simply growing pigmented filamentous callus inside the agar (embedded callus culture). Further, addition of plant growth regulators (NAA and BAP) enhanced the process of somatic embryogenesis in subcultured pigmented filamentous callus.

5. The growth rate of tissue culture progeny in field cultivation was not clearly defined and did not report of fast growing strains.

However, in the instant Application the plants developed from tissue culture studies showed improved growth rate over control ones even after two years in field. The plants developed from tissue culture studies showed improved growth rate over control ones even after two years in field.

"C. J Dawes and E.W. Koch (*J. Appl. Phycology*, 3, 247-257, 1991) and C. J.Dawes, G. C. Trono and A. O. Lluisma (*Hydrobiologia*, 260/261, 379 - 383, 1993) made attempts to

develop suitable methods for maintenance and propagation of selected clones of different cultivated varieties of *Eucheuma* through micro-propagation and tissue culture. Their studies laid more emphasis on establishment of suitable laboratory culture techniques for clonal propagation of farmed *Eucheuma* using tiny vegetative fragments. The main drawback of such propagation using micro-cuttings is that the progeny will possess only parental features and does not have a pronounced advantage over the parental populations in expression of desired traits."

They do not show any variation between the tissue culture raised plantlets versus the normal grown algae. They have also not produced somatic embryos by tissue culture whereas in I invention I have gone through this step of somatic embryogenesis, this has let to larger number of plantlets raised through tissue culture, as somatic embryogenesis is a rapid method for propagation.

"O. P. Mairh *et al.* (*Indian J. Marine Sciences*, 24, 24 - 31, 1995) successfully demonstrated the feasibility of bag cultivation of *Eucheuma striatum* on experimental scale in field conditions. However, the main drawback of this method is that this resulted in decrease in their daily growth rate as compared to those grown in open waters without polythene bags." They have not used tissue culture raised plantlets and they have not used it in the open sea conditions.

The present invention reveals the development of fast growing strains of algae under *in vitro* conditions and development of the method of producing micropropagules on a large scale through somatic embryogenesis of pigment callus of anatomically complex thallophytic red algae. The plantlets obtained through germination of somatic embryos have several advantages namely, they are faster in growth and they produce variability as compared to the parent material.

It also deals with the development of the clone with improved trait, which has not been shown by Dawes et al. (1991).

The above referred work of Dawes et al. 1991 as stated in the prior art of the application mainly focuses on establishment of inexpensive *in vitro* culture facility near the farming areas for maintenance of seed stock of *Eucheuma* using mostly branches and micropropagules (vegetative cuttings of 1-5mm segments, e.g. page 248 and Fig.3 and page 253 and Table 2B) in liquid cultures. For achieving this, they have made various possible approaches including selection of source material from various parts of thallus, development of methods for aseptic culture, optimization of liquid culture medium, culture conditions and influence of various plant growth regulators etc. All the above conditions optimized for *Eucheuma* by Dawes et al. 1991 are mostly for branch and micropropagules (vegetative cuttings of 1-5mm segments, e.g. page 248 and Fig.3 and page 253 and Table 2B) culture and not for callus induction.

Though they have reported success in developing callus and subsequently regeneration of plantlets in *Eucheuma*, it does not report about subculture of excised pigmented callus on solid medium (Fig.2), development of tiny micropropagules directly from subculture

pigment callus (Fig.3), callus (Fig.6) and regeneration of somatic embryos (Fig.4 and 5), which are among the main inventory steps of present patent application.

Dawes et al. 1991 also reported that cloning of branch segments to produce micropropagules. Therefore, the micropropagules described by Dawes et al. 1991 is basically vegetative micro-cuttings of plant.

Mairh et al. 1995 teaches culture of *Kappaphycus striatum* in laboratory, in tanks and in field (tide pools in intertidal zone) using fragments (apical, middle and basal) incised obliquely, transversely and longitudinally. His study never described tissue culture of *Kappaphycus striatum* as stated by Examiner. Moreover, Mairh et al. 1995 carried out cultivation experiments using polythene bags in tide pools for a shorter period. The present patent application describes a method for large-scale cultivation in open sea using polythene bags.

In this invention I have demonstrated entirely a new method of growing excised pigmented filamentous callus of *Eucheuma* species as "embedded culture" method (claim 1(d)), which resulted in large-scale production of micropropagules (tiny spherical or oval shaped particles of 2-5mm diameter) through somatic embryogenesis of filamentous callus.

C. J Dawes and E.W. Koch (*J. Appl. Phycology*, 3, 247-257, 1991) and C. J. Dawes, G. C. Trono and A. O. Lluisma (*Hydrobiologia*, 260/261, 379 - 383, 1993) made attempts to develop suitable methods for maintenance and propagation of selected clones of different cultivated varieties of *Eucheuma* through micro-propagation and tissue culture. Their studies laid more emphasis on establishment of suitable laboratory culture techniques for clonal propagation of farmed *Eucheuma* using tiny vegetative fragments. The main drawback of such propagation using micro-cuttings is that the progeny will possess only parental features and does not have a pronounced advantage over the parental populations in expression of desired traits.

O.P. Mairh *et al.* (*Indian J. Marine Sciences*, 24, 24 - 31, 1995) successfully demonstrated the feasibility of bag cultivation of *Eucheuma striatum* on experimental scale in field conditions. However, the main drawback of this method is that this resulted in decrease in their daily growth rate as compared to those grown in open waters without polythene bags.

The present invention reveals for the first time development of fast growing strains under *in vitro* conditions as lll as a method of producing micro-propagules clonally on large scale through somatic embryogenesis of pigmented callus of anatomically complex thallophytic red alga. The earlier studies on tissue culture of seaweeds demonstrated the regeneration of entire plants *de novo* by transferring the callus to liquid medium. But in the present invention for the first time succeeded in producing somatic embryos like pigmented micro-colonies consisting of cells from as little as three to several hundred on agar plate itself. I found that the pigmented micro-colonies on agar plates under dim light conditions can ideally be preserved live for extended periods till needed. The daily

growth of field grown plants raised from tissue culture have, in several cases, exhibited more than 40% increase over the control plants under similar conditions, At the end of 60 days, this translates to a two-fold increase in biomass over control which is more or less similar to that obtained for open waters. The semi-refined carrageenan yield from dry tissue culture raised plants is 43 % and gel strength is 540 g. cm $^{-2}$ while for the control parent plant s the yield is 43 % and gel strength of the carrageenan is 550 g. cm $^{-2}$.

The inventive steps adopted in the present invention are (i) development of a methodology for obtaining a clean and axenic plant material for tissue culture, (ii) development of fast growing variants, with two-fold increase in growth without change in carrageenan yield and quality, through micro-propagules of pigmented callus, (iii) *in vitro* clonal propagation of micro-propagules through somatic embryogenesis of pigmented callus by growing as embedded cultures inside the agar plates, (iv) stimulation of process of formation of somatic embryos in pigmented callus using plant growth regulators such as naphthalenacetic acid and 6-benzylaminopurine and (v) cultivation of algae in an enclosed transparent plastic bags with perforations, which prevents grazing and provides pure raw material, free of contaminants likely to affect adversely the final processed product.

More specifically, the improved cultivation process includes the methodology for development of a clone with an improved trait, i.e., faster growth, through tissue culture and cultivation by long line floating method using a transparent polythene bag with small perforations, which prevents grazing and provides pure raw material, free of contaminants which are likely to affect adversely the final processed product kappa carrageenan (κ-carrageenan).

The main usage(s)/ utility of the improved cultivation method includes the following:

(i) methodology for development of soma clones with an improved trait, i.e., faster growth, through tissue culture, (ii) *in vitro* clonal propagation of seaweeds through somatic embryogenesis of callus cells, (iii) production and supply of uniform seed stock (micropropagules) on large scale through *in vitro* somatic embryogenesis of callus culture for practical farming of seaweeds, (iv) exploitation of the callus as a source for long term storage of germplasm, and (v) cultivation in a transparent polythene bag with small perforations, which prevents grazing and provides pure raw material, free of contaminants detrimental to the processed product, κ-carrageenan.

Comparative study of growth, carrageenan yield and gel properties of tissue culture progeny and control plants: A comparative study on growth (Fig.8), carrageenan (semi refined) yield on dry seaweed light basis, and carrageenan gel strength of field grown plants raised from tissue culture progeny and control parent plants was carried out. Both type of plants Ire grown in 10 replicates in closed transparent polythene bags by floating long line method as described earlier in Example 6. An average biomass of 394.58 $\pm$ 20.8 g. fresh light (4.7% daily growth rate) was obtained for 30 days and 846.6 $\pm$ 38 g. fresh light (3.5% daily growth rate) for 60 days, while tissue culture

plants had 711.6 ± 13 g. fresh light (6.8% daily growth rate) for 30 days and 1590 ± 37 g. fresh light 4.6% daily growth rate) for 60 days period.

The main advantages of the present invention are: A. A new method of improving a parent plant to introduce improved traits such as fast growth. B. A method of producing large number of micropropagules (seed material) rapidly from desired strains. C. A means of storage of germplasm in viable form, i.e., as somatic embryos, on agar plates. D. An improved method of cultivation of seaweeds which reduces adverse impact of the vagaries of nature such as strong water current and damage to crops by grazers and epiphytes and yet allows the same or better growth rate than what is realized with the parent plant in open waters. E. An improved method of cultivation, which provides material of highest purity free from all contaminants detrimental to the quality of final processed end product.

Dawes et al. and Mairh et al., either alone or in combination, fail to teach or suggest the claimed invention, as required by claim 1. Particularly, the cited references do not disclose a tissue culture obtained by sub-culturing thin slices of pigmented callus as an embedded culture in agar plates thereby further enhancing micropropagules production. Also, there is no clue to do it to achieve better results. So, how can the invention be obvious? None of the cited arts make any reference to the fact that sub-culturing thin slices of pigmented callus as an embedded culture in agar plates would further enhancing micropropagules production. So, it is unreasonable on the part of the Examiner to suggest that it is obvious.

Further, the cited references do not teach using the enhanced micropropagules as obtained above to cultivate plantlets in enclosed bags in open sea conditions. Dawes et al. discloses nothing on enhanced micropropagules production in obtaining its tissue culture, namely sub-culturing thin slices of pigmented callus as an embedded culture in agar plates.

Further, Mairh et al. fails to remedy the deficiencies of Dawes et al. In fact, Mairh et al. merely discusses bag cultivation possibilities and particularly the culture of *Kappaphycus striatum* in a laboratory, in a tank and in the field (tide pools in the intertidal zone) using apical, middle and basal fragments incised obliquely, transversely and longitudinally. Mairh et al. does not disclose sub-culturing thin slices of pigmented callus as an embedded culture in agar plates and does not disclose cultivation in the open sea. For at least these reasons, Dawes et al. and Mairh et al. do not teach the features of the claim 1.

Furthermore, as the combination of Dawes et al. and Mairh et al. fails to teach the features of claim 1, there is no suggestion that combining the references could lead to any advantages enjoyed by the claimed invention. The present invention provides an advantageous process of algae cultivation wherein a high yielding tissue culture plant clone is used for growing in a bag to produce a biomass as much as that conventionally obtained in open waters without a bag. Even if the teachings of Dawes et al. and Mairh et al. could be combined, which Applicants do not concede, the references fail to derive the features of claim 1 and would not lead to the unexpected results achieved by the claimed

invention. Accordingly, it is respectfully submitted that claim 1 and dependent claims there from are patentable over Dawes et al. and Mairh et al.

The hindsight study and splitting of the invention into parts and identifying one prior art for each part is not the appropriate way of scrutinizing the patentability of the subject matter. This is not the manner of doing research work. If the strategy for linking-up of the parts of the invention after splitting one invention were appropriate, then it would be impossible to get a patent.

It might appear extremely simple to conduct the invention once you know about it. However, the real challenge is to be the first to do it. Had it been so simple and easy to achieve the results of the instant Application, then, it would have done long back. However, that is not the case. It has taken the inventors considerable amount of time and effort to achieve these results.

Further, the inventions in the field of life sciences are not as predictable as it is in other fields of sciences. One cannot assume things just like that. The life systems might behave very different under a slightest of change in the environment. Unless one actually does the work, it cannot be conclusive simply on the basis of imagination. If extrapolation of all the previous knowledge was sufficient to obtain the results, then, we do not need any of these laboratories and research institutes. It is a well-known fact that mere change in the levels of plant hormones in the medium or change in the duration of exposure to sunlight would lead to surprisingly different results. Now, if the results are so varied then, how can it be obvious? The life sciences do not follow the law of uniformity and thus, one cannot be sure unless the experimentation is actually conducted in the laboratory or in the fields.

Thus, I strongly feel that the instant Application is non-obvious and thus, patentable.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements Ire made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardise the validity of the application or any patent issued thereon.

Signature: _____

(Kalpana Harish Mody)

Date: 30/06/2003